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Inhibition of osteoblast activity by zoledronic acid

Inibição da atividade de osteoblastos expostos ao ácido zoledrônico

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ABSTRACT

Introduction: Patients treated with nitrogen-containing bisphosphonates, such as zoledronic acid (ZA), have frequently shown oral bone exposure areas, termed osteonecrosis. In addition, these patients may also present low repair and regeneration potential, mainly after tooth extractions. These side-effects caused by bisphosphonates may be due to their inhibitory effects on oral mucosa and local bone cells. **Objective:** To evaluate the effects of ZA on the mineralization capacity of cultured osteoblasts. **Materials and methods:** Human immortalized osteoblasts (SaOs-2) were grown in plain culture medium (Dulbecco's Modified Eagle Medium [DMEM] + 10% fetal bovine serum [FBS]) in wells of 24-well plates. After 48-hour incubation, the plain DMEM was replaced by a solution with ZA at 5 µM which was maintained in contact with cells for seven, 14 or 21 days. After these periods, cells were evaluated regarding alkaline phosphatase (ALP) activity and mineral nodule formation (alizarin red). Data were statistically analyzed by Mann-Whitney test, at 5% of significance level. **Results:** ZA caused significant reduction on ALP activity and mineral nodules formation by cultured osteoblasts in all evaluated periods ($p < 0.05$). **Conclusion:** These data indicate that ZA causes inhibition on the osteogenic phenotype of cultured human osteoblasts, which, in turn, may reduce bone repair in patients subjected to ZA therapy.

Key words: osteonecrosis; repair; mineralization; osteoblasts.

INTRODUCTION

Bisphosphonates are indicated for the treatment of diseases characterized by intense bone resorption. Their main mechanism of action is inhibition of osteoclast maturation and activity. The use of these drugs has been linked to the development of osteonecrosis in the oral cavity, a condition that seems to be associated with the reduction of tissue repair capacity[2, 3].

Several studies demonstrate that bisphosphonates present inhibitory effect on different cell types, like osteoblast and epithelial cells, what may explain, at least partially, the development of osteonecrosis[1, 11, 14].

The zoledronic acid (ZA) is known as a potent bisphosphonate, particularly due to its greater capacity of adherence to the bone tissue[9]. It is administered intravenously, with the frequency of use varying according to the disease to be treated and the established therapy[9]. Patients in treatment with this kind of bisphosphonate have presented higher risk for the development of osteonecrosis, what is directly related to the dose and the frequency of use[9].

The etiopathogenesis of bisphosphonate-induced osteonecrosis has been associated with local traumas, like tooth extractions[6]. Besides, individuals treated with ZA also present low capacity for tissue repair and regeneration. This fact would explain the delay in the repair of bone and mucous tissue in patients treated with this drug[3,10].

The low repair capacity after tooth extractions observed in patients treated with bisphosphonates may be related to a reduction in mineralization activity by local osteoblasts. Therefore, the objective of this study was to assess the mineralization capacity of osteoblasts exposed to ZA.

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MATERIALS AND METHODS

Cell culture

An immortalized human osteoblast cell line was used (SaOs-2 ATCC HTB-85). The cells, grown in plain culture medium (Dulbecco's Modified Eagle Medium [DMEM], GIBCO, Grand Island, NY, USA), containing antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin – GIBCO) and 10% fetal bovine serum ([FBS] – GIBCO), were kept in incubation at 37ºC with 5% CO_2, until the obtainment of the necessary number for the conduction of the experiment.

Thus, cells were seeded in wells of 24-well plates (Techno Plastic Products AG, Trasadingen, Switzerland), for 48 hours. Next, the complete DMEM was replaced by an osteogenic differentiation medium, characterized by the presence of ß-glycerophosphate and ascorbic acid (Sima-Aldrich, St. Louis, MO, USA), but without the addition of FBS. Five µM ZA (Novartis, São Paulo, SP, Brazil) were added to this new medium, which was put in contact with cells during seven, 14 and 21 days. The osteogenic medium was replaced every three days, with new addition of the drug, in the same concentration.

Alkaline phosphatase (ALP) activity

Cells exposure to the solution of ZA in the defined periods was followed by the assessment of alkaline phosphatase (ALP) activity, which is highly expressed by osteoblasts and takes part in the mineralization of the bone matrix. This assessment was conducted as described by Oliveira *et al.*[7]. In general, the used protocol characterizes as a colorimetric endpoint assay, which produces, by means of a dephosphorylation reaction of thymolphthalein, a bluish solution, which may come in different shades according to the activity of the ALP enzyme present in the solution. This enzyme activity was determined spectrophotometrically (Termo Plate, Nanshan District, Shenzhen, China) by absorbance reading of the 590-nm solution. Its concentration is determined from a standard curve prepared according to the analysis of the known ALP concentrations.

Formation of mineralized nodules (alizarin red)

Osteoblast capacity to form mineralized nodules in culture was accomplished according to Idris *et al.*[5]. This assay reflects the capacity of these cells to form an organized and mineralized matrix, typical of bone tissue in the process of repair.

For the implementation of this protocol, cells were cultured in osteogenic differentiation medium during the experimental process (seven, 14 and 21 days). This medium provides the necessary substrates for the synthesis and deposition of components of the bone matrix by osteoblasts in culture.

After fixation in etanol 70% at 4ºC and staining with alizarin red (40 mM, pH 4.2) (Sigma-Aldrich, St. Louis, MO, USA), it was possible to visualize the formation of mineralized nodules under an optical microscope (Olympus BX51, Miami, FL, USA), attached to a camera (Olympus C5060, Miami, FL, USA), for a qualitative analysis.

Part of the samples was subjected to quantitative analysis of mineralized nodule formation, which consists of the dissolution of the nodules in a cetylpyridinium chloride solution (Sigma-Aldrich). This dissolution results in the formation of a homogeneous solution, which is subjected to a spectrophotometric (Termo Plate) analysis of absorbance (562 nm).

Statistical analysis

Data on ALP and formation of mineralized nodules were assessed for normality. As these data did not present normal distribution, Mann-Whitney test was used, considering a significance level of 5%.

RESULTS

Data on the ALP activity by osteoblasts (SaOs-2), according to treatment and assessment period, are presented on **Table 1**.

TABLE 1 – Alkaline phosphatase activity (%) of SaOs-2 cells, according to treatment and assessment period

Period (days)	Treatment	
	Control	Zoledronic acid
7	*101.51 (92.81-108.74) a A	37.37 (32.90-46.74) b A
14	99.04 (95.87-105.05) a A	16.86 (16.62-17.33) b B
21	101.24 (94.55-103.05) a A	19.51 (18.76-20.69) b B

Values are medians (25th percentile-75th percentile), n = *8.*
**Lower case letters allow the comparison in the lines; capital letters, in the columns. Values with the same letters do not differ statistically (Mann-Whitney,* p > *0.05).*

For the period of seven days, the group treated with ZA presented reduction of 64% in the ALP activity values when compared to the control group with no treatment ($p = 0.001$). In later periods, of 14 and 21 days, the same pattern of cellular response was observed, with an 82% reduction of ALP activity ($p = 0.001$) for both groups.

There were no differences in ALP activity for the control group in the three assessment periods ($p > 0.05$). For the group treated

with ZA, the period of seven days presented the highest ALP values when compared to the other periods, with significant statistical difference ($p < 0.05$). The periods of 14 and 21 days did not differ from each other ($p > 0.05$).

The data on mineralized nodule formation by SaOs-2 cells, according to treatment and assessment period, are presented on **Table 2**.

TABLE 2 – Formation of mineralized nodules (%) of SaOs-2 cells, according to treatment and assessment period

Period (days)	Treatment	
	Control	Zoledronic acid
7	*98.42 (96.40-105.69) a C	13.39 (11.58-21.23) b C
14	110.04 (107.40-114.53) a B	27.09 (18.91-37.08) b B
21	107.26 (106.09-120.89) a A	43.95 (39.17-47.96) b A

Values are medians (25th percentile-75th percentile), n = *8.*
**Lower case letters allow the comparison in the lines; capital letters, in the columns. Values with the same letters do not differ statistically (Mann-Whitney,* p > *0.05).*

Cells treatment with ZA decreased mineralized nodule formation. In the period of seven days, the treated group suffered an 85% reduction on the values of mineralized nodule formation when compared to the control group ($p = 0.001$). In the periods of 14 and 21 days, the reductions were 83% and 63%, respectively ($p = 0.001$).

When comparing the different assessment periods, the period of 21 days presented the highest values in nodule formation, followed by the periods of 14 and seven days, with a statistical difference among all of them ($p < 0.05$).

DISCUSSION

The results of this study show that human osteoblasts exposed to ZA presented a significant reduction in the capacity to produce components of matrix formation and mineralization. This was determined by the significant reduction of ALP activity by osteoblasts, which had their capacity to form mineralized nodules dramatically reduced. Within the limitations of the present study, one may suggest that these scientific data observed in cultured human osteoblasts could explain, at least partially, Allen's[3] results. He demonstrated delay in bone repair in patients treated with this medicine.

Orris *et al.*[8] also studied the activity of human osteoblasts in culture, however the concentrations of ZA used by the authors were 10 nm, 100 nm, 1 µM and 100 µM, which did not correspond to those found in the oral cavity of patients treated with this kind of bisphosphonate[12]. Despite the methodological differences, Orris *et al.*[8] found results similar to those in this study, particularly related to the significant reduction in ALP and mineralized nodule production, which presented as dose- and time-dependent. Besides, previous studies demonstrated that ZA also promotes significant inhibition of the expression of type I[6, 13] collagen, main organic component of the bone matrix, what directly interferes in the process of tissue repair[4].

By knowing the possible undesirable side-effects of ZA on the metabolism of different cell types and the resulting interference of this drug in the repair and regeneration process, perhaps other types of bisphosphonates might be used more safely. However, Idris *et al.*[5] showed that other aminobisphosphonates, despite seeming less potent than ZA, also caused important negative effects on the activity of osteoblasts *in vitro*, as it was demonstrated in this and in previous studies[6, 8, 12, 13].

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RESUMO

Introdução: *Pacientes em tratamento com bisfosfonatos, sobretudo com os nitrogenados, como o ácido zoledrônico (AZ), têm apresentado áreas de exposição de tecido ósseo na cavidade oral, caracterizadas como osteonecrose. Essas áreas apresentam limitada capacidade de reparo, principalmente após exodontias. Esses efeitos podem ser resultado do efeito inibitório causado por esse tipo de medicamento sobre as células da mucosa e do tecido ósseo local.* ***Objetivo:*** *Avaliar o efeito do AZ sobre a capacidade de mineralização de osteoblastos* in vitro*.* ***Materiais e métodos:*** *Foi utilizada uma linhagem celular de osteoblastos humanos (SaOs-2). As células foram cultivadas em meio de cultura completo (Dulbecco's Modified Eagle Medium [DMEM] + 10% de soro fetal bovino [SFB]). Após 48 horas de incubação, o DMEM completo foi substituído por um novo DMEM sem SFB, ao qual foram*

*adicionados 5 µM de AZ. Essa solução foi mantida em contato com as células por sete, 14 ou 21 dias. Após esse período, as células foram avaliadas quanto à atividade de fosfatase alcalina (ALP) e à produção de nódulos de mineralização (*alizarin red*). Os dados foram submetidos ao teste estatístico de Mann-Whitney, considerando nível de significância de 5%.* **Resultados:** *A exposição das células ao AZ causou redução significativa na atividade de ALP e na formação de nódulos mineralizados em todos os períodos avaliados (*$p < 0,05$*).* **Conclusão:** *Esses resultados revelam que o AZ causa alteração inibitória no fenótipo osteogênico das células humanas em cultura, o que pode reduzir a capacidade de reparo do tecido ósseo após o contato com esse tipo de medicamento.*

Unitermos: *osteonecrose; reparo; mineralização; osteoblastos.*

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